The proteolytic activity of benthic bacteria in three estuarine lakes

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Estuaries
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ZBIGNIEW MUDRYK
Department of Botany and Microbiology,
Pedagogical University,
Słupsk

WOJCIECH DONDERSKI
Department of Water Microbiology,
Nicolaus Copernicus University,
Toruń

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Abstract

Investigations to determine the number and proteolytic activity of bacteria inhabiting the bottom sediments of three estuarine lakes are reported. Proteolytic bacteria occurred in large numbers in all the lakes. Proteolytic activity differed depending on the time of sampling and whether the bacteria were halophilic or nonhalophilic. Bacteria isolated from the hypertrophic Lake Jamno demonstrated a greater proteolytic activity than those from the eutrophic Lakes Łebsko and Gardno.

1. Introduction

Estuaries are dynamic environments in which the chemical and biological gradients are highly variable. In comparison with freshwater and marine basins, estuarine ecosystems exhibit relatively high rates of primary and secondary production (Means and Wijayarante, 1984). Estuarine lakes thus contain large quantities of chemically diverse organic matter. Its dominant component is protein, constituting 35–55% of dry mass (Little *et al.*, 1979). Occurring abundantly in estuaries, heterotrophic bacteria play an essential role in the decomposition of these compounds. Because bacteria are incapable of directly taking up large biopolymers such as proteins, these must first be hydrolysed by extracellular proteases into monomeric subunits, mainly amino acids. These are then very rapidly taken up by bacteria and can be catabolized and respired or used for biosynthesis (Sizemore *et al.*, 1973;

Billen *et al.*, 1980; Fuhrman and Ferguson, 1986). The object of the present study was to determine the abundance and the enzymatic activity of the proteolytic strains of bacteria isolated from the sediment in order to acquire information on the potential destruction of proteins in the bottom sediments of three estuarine lakes.

2. Material and methods

The study was carried out in three estuarine lakes: Łebsko, Gardno and Jamno (Fig. 1). These lakes are extensive, with surface areas ranging from 2430 to 7440 ha, but are very shallow (average depth – 1.3–1.7 m). Narrow channels connect them with the Baltic Sea. Large volumes of sea water flow back through these channels into the lakes during gales with a northerly component, particularly in spring and autumn. A number of characteristic physicochemical parameters of the lakes are set out in Table 1 (Trojanowski, *et al.*, 1991).

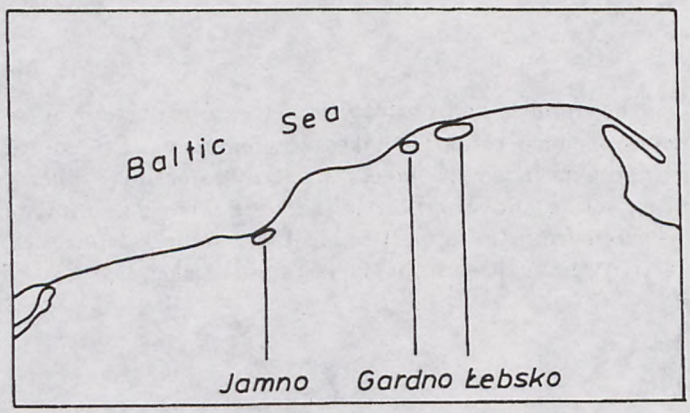

Fig. 1. Location of the three lakes studied

Bottom sediments were collected from each lake with a pipe sampler at three-month intervals. The upper layer sediments (to a depth of 10 cm) were transferred aseptically into sterile glass bottles, placed in an ice-filled container and then transported to the laboratory. The time between sampling and analysis did not usually exceed 4–6 hours.

For determining the total number of heterotrophic bacteria, including proteolytic organisms, the bottom sediment samples were diluted with sterile buffered water (Daubner, 1967) and inoculated by the spread plate method on iron-peptone agar (IPA) prepared according to Ferrer *et al.*, 1963). After 10 days' incubation at 20°C the bacterial colonies were counted and

Table 1. Mean values of selected physicochemical parameters of the water in Lakes Jamno, Gardno and Lebsko in the period 1986-1989

Lake	pH	BOD_5 mg O_2	Secchi disc visibility m	NH_4 – N mg N·dm^{-3}	N — total mg N·dm^{-3}	P — total mg P·dm^{-3}	Chlorophyll *a* mg·m^{-3}
Jamno	9.1	9.6	0.25	0.5	5.9	0.5	128.4
Gardno	9.0	6.6	0.35	0.1	3.7	0.4	85.3
Lebsko	8.8	4.9	0.57	0.2	3.2	0.3	68.5

recalculated per gram of dry sediment weight. Then, from the whole surface of the plates or from a particular sector, 100 bacterial colonies from each sample were picked out and transferred to a semisolid IPA medium (5.0 g agar per litre). After purity control the strains were stored at 4°C and inoculated on fresh medium every 3 months.

The ability of the bacteria to hydrolyse proteins was determined in IPA medium containing 20.0 g gelatin per 1 dm^3. After six days' incubation at 20°C the proteolytic bacteria were detected on the basis of a clear zone appearing around the colonies flooded with Frazier's reagent. In further studies to determine proteolytic activity, strains were used whose clear zone around the colonies was larger than 20 mm. Moderately halophilic strains were sought among these bacteria. For this purpose, bacteria were incubated at 20°C for six days on a liquid medium prepared according to Jones (1971), with 50.0 g·dm^{-3} sodium chloride added. The intensity of bacterial growth in the test medium was measured in a Specol photocolorimeter at 600 nm with an ER-1 countershaft.

Bacteria whose growth, determined by light penetration, was less than 50% were taken to be moderately halophilic, after Kushner (1968) and Larsen (1981). In order to determine the proteolytic activity, 20 bacterial strains (10 nonhalophilic and 10 moderately halophilic) were selected from each lake. These strains were grown on IPA medium slants at 20°C for 72 hours. The bacteria were then washed off with buffered water (Daubner, 1967) and the optical density of the suspension adjusted to E = 0.3 at 600 nm using a Specol photocolorimeter. After this, 0.5 cm^3 of the suspension was introduced into Erlenmeyer flasks each containing 25 cm^3 of the following medium: 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.1 g ferrous gluconate, 0.1 g yeast extract (Difco), 2.5 g peptone (Proteose Difco), 2.5 g casein (Casein fat free, BDH), 1 dm^3 tap water (pH 7.2). The bacterial cultures were conducted in duplicate at 20°C for 24 hours on a rotary shaker. The proteolytic activity determination employed the crude enzyme containing the supernatant obtained by centrifuging the culture at 16000 × g for 30 minutes. The reacting mixture contained 1 cm^3 culture supernatant, 1 cm^3 2% casein according

to Hammersten (Fluka) in 0.06 M phosphate buffer (pH 7.0) and 2 cm^3 of the appropriate buffer. The enzymatic reaction was allowed to proceed at 37°C for one hour, then stopped by adding 4 cm^3 10% trichloroacetic acid. The reacting mixture was left at room temperature for 30 minutes, then filtered through Whatmam No. 42. The amount of tyrosine released during the enzymatic reaction was determined according to Schacterle and Pollack (1973) and Donderski (1988) using phenol and copper reagents (Folin – Ciocalteu). Measurements were made at 650 nm in the Specol photocolorineter. The proteolytic activity of bacteria was expressed in μg of tyrosine per 1 cm^3 of culture per hour or in μg tyrosine per 1 mg enzymatic protein contained in the cell-free supernatant per hour. The amount of protein in the cell-free supernatant was determined according to Schacterle and Pollack (1973) using bovine albumin (BDH) as standard.

3. Results

The results given in Table 2 indicate that proteolytic bacteria occurred in large numbers in the bottom sediments of all three estuarine lakes, making up 48–96% of the total number of strains examined. The numbers of protein-hydrolysing bacteria reached a maximum in Lakes Łebsko and Gardno in spring, but in Lake Jamno in winter. In all three lakes, the percentage of proteolytic bacteria was lowest in autumn.

The data presented in Table 3 and Figure 2 indicate pronounced differences in the levels of benthic bacterial activity, both in particular seasons of the year and between the halophilic and nonhalophilic bacteria.

The highest mean proteolytic activity of all the studied strains was found among the bacteria isolated from Lakes Łebsko and Gardno in autumn, and from Lake Jamno in winter. In Łebsko and Gardno this activity was low during summer, whereas in Jamno it remained at a fairly high, only slightly varying, level throughout the whole investigation period.

The proteolytic activity of halophilic baceria was generally higher in Łebsko and Jamno. Particularly the strains isolated in winter showed a great ability to release proteolytic enzymes. In Gardno, however, the nonhalophilic bacteria were more enzyme-active than the halophilic ones throughout the study period.

Figure 2 also suggests that proteolytic activity was higher in bacteria from the hypertrophic Lake Jamno than in those isolated from the eutrophic Lakes Łebsko and Gardno.

Table 2. Number of colony forming units (millions per gram of dry sediment) in the bottom sediments of Lakes Łebsko, Gardno and Jamno

Date of sampling	Lake Łebsko		Lake Gardno		Lake Jamno	
	Hetero-trophic bacteria	Proteolytic bacteria	Hetero-trophic bacteria	Proteolytic bacteria	Hetero-trophic bacteria	Proteolytic bacteria
05.1988	3.58	2.86 (80)	34.37	33.10 (96)	93.82	53.01 (56)
07.1988	8.89	6.17 (69)	38.71	27.45 (71)	147.21	108.41 (74)
10.1988	5.72	3.21 (56)	21.18	14.12 (67)	109.16	52.33 (48)
03.1989	1.43	0.89 (62)	–	–	48.72	41.39 (85)

Explanations: In parentheses – percentage of proteolytic bacteria

Table 3. Proteolytic activity of bacterial isolates from the bottom sediments of Lakes Łebsko, Gardno and Jamno (values are means for the twenty strains; range of estimates in brackets)

Date of sampling	Lake Łebsko		Lake Gardno		Lake Jamno	
	Activity					
	specific+	total++	specific	total	specific	total
05.88	121.7(76.7-168.2)	18.4(12.0-30.6)	135.3(59.4-283.7)	23.9(4.8-58.9)	160.2(77.0-295.8)	13.6(4.4-36.6)
07.88	49.8(16.8-97.2)	11.7(0-26.0)	31.3(13.0-48.1)	8.6(2.5-13.4)	134.5(55.9-255.0)	14.7(0-26.9)
10.88	153.3(116.6-198.7)	11.9(0-19.1)	151.9(99.1-173.4)	13.8(0-20.0)	117.2(43.8-197.6)	13.4(7.5-19.6)
03.89	127.6(48.7-253.6)	16.4(7.2-27.2)	–	–	183.4(72.6-373.1)	31.9(0-96.3)

Explanations: + – specyfic activity = μg of tyrosine released by 1 mg protein enzymes per hour
++ – total activity = μg of tyrosine released by 1 cm^3 of culture (cell free medium) per hour

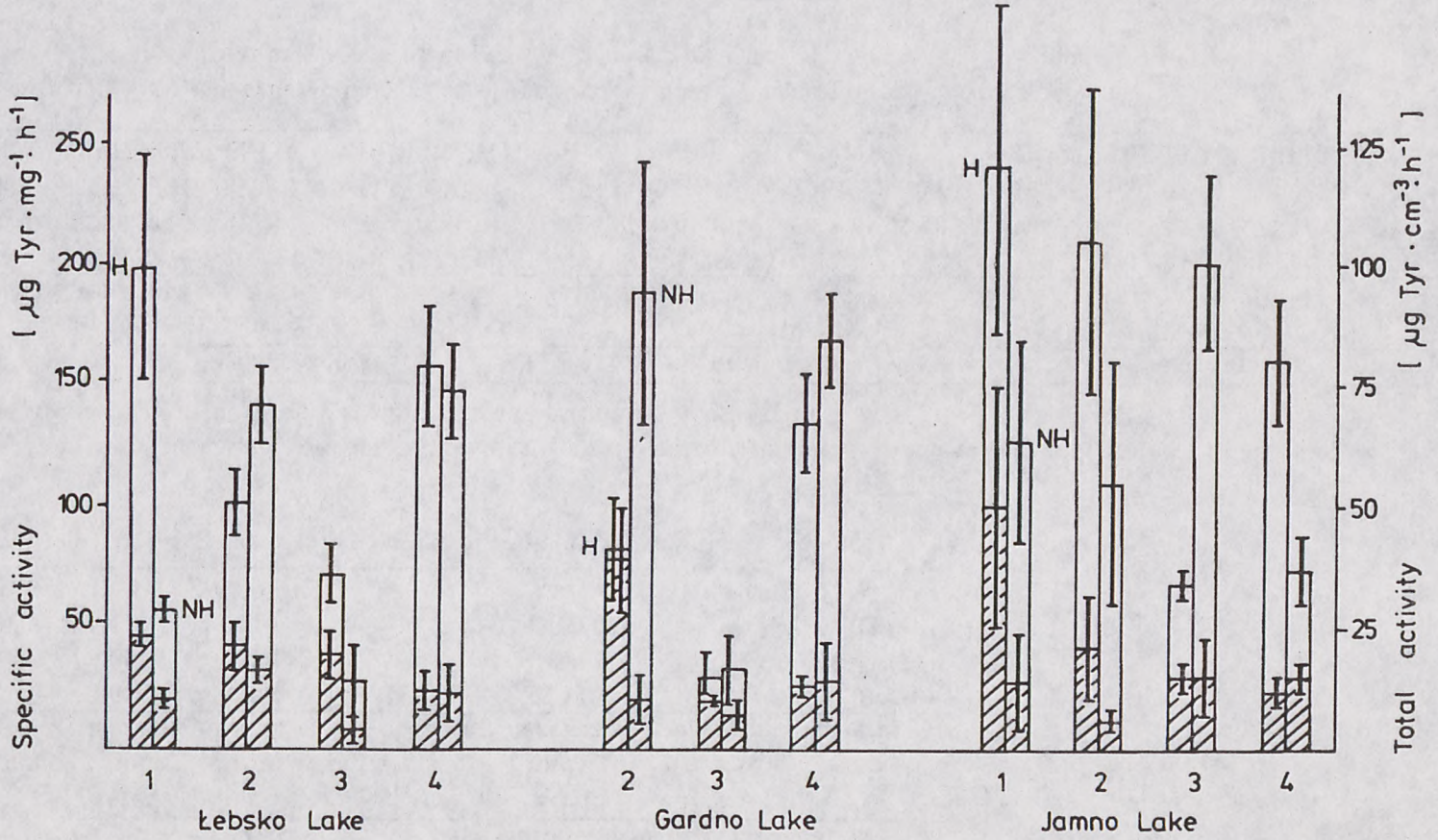

Fig. 2. Average specific and total proteolytic activity of bacteria isolated from the bottom sediments of the investigated lakes. Vertical bars represent standard deviation

□ specific activity, ▨ total activity, H – halophilic bacteria, NH – nonhalophilic bacteria, 1 – winter, 2 – spring, 3 – summer, 4 – autumn

4. Discussion

Proteolysis, *i.e.* the enzymatic decomposition of proteins, is one of the most important processes affecting the nitrogen and carbon cycles in aquatic environments (Reichardt, 1988). That is why proteolytic bacteria occur in large numbers in many water basins, including estuaries. They may constitute 70–100% of the total number of heterotrophic bacteria (Hashimoto *et al.*, 1983; Mow-Robinson and Rheinheimer, 1985). In the estuarine lakes studied by us, the organisms able to hydrolyse proteins also made up a large percentage of the benthic bacteria (48–96%). Their occurrence was subject to distinct seasonal variations. Bacteria of this physiological type were most numerous in the winter-spring season. These results are in agreement with those obtained by Sizemore *et al.* (1973) for the bottom sediments of Debidue Creek.

Reichardt (1986) found a distinctly positive correlation between the number of protein-hydrolysing bacteria and proteolytic activity in Kiel Bight bottom sediments; the numerous populations of heterotrophic bacteria inhabiting water basins exhibit a capacity for synthesizing proteases (Little *et al.*, 1979). Both the intensity of this synthesis and the regulation of the activity of such enzymes may be influenced by environmental factors like temperature, pH, oxygen concentration, salinity and hydrostatic pressure (Weimer and Morita, 1974; Hare *et al.*, 1981), The positive effect of higher temperatures on the activity of extracellular proteolytic enzymes, reported by some authors (Juffs, 1976; Mow-Robinson and Rheinheimer, 1985; Donderski, 1988), was not confirmed in the present study. We observed the highest activity of proteolytic enzymes in the strains isolated during the autumn – winter season; in summer, however, *i.e.* the period of highest temperatures, the activity of proteases synthesized by strains isolated from Lakes Łebsko and Gardno was lowest. Identical results were obtained by Meyer-Reil (1983) for the bottom sediment microflora of the Kiel Bight and by Little *et al.* (1979) for Lake Champlain. These data suggest that the enzymatic activity of bacteria is influenced not only by temperature but probably also by the concentration of the hydrolysed substrate as well as by the ability of bacteria to adapt physiologically to different environmental temperatures (Sieburth, 1967; Mow-Robinson and Rheinheimer, 1985).

Reichardt (1987) stated that protease activity often remains high at temperatures below 20°C and the results obtained by Sarayuma *et al.* (1980) indicate that at low temperatures the synthesis of proteases may even increase, especially in psychrophilic bacteria.

In Lakes Łebsko and Jamno the proteases synthesized by halophilic bacteria displayed greater activity than those synthesized by nonhalophilic organisms. Gibbons (1957) and Gutierez and Gonzales (1972) stated

that about 90% of halophilic bacteria are capable of synthesizing proteases. Gutierez and Gonzales (1972) point out that for optimum growth halophilic bacteria use proteins more intensively than carbohydrates. Hence they have a very well developed and efficiently functioning complex of proteolytic enzymes, ensuring their active assimilation of amino acids. The highest activity of these enzymes is observed at a relatively low salinity (< 0.4%) (Weimer and Morita, 1974). This may account for the marked proteolytic activity of halophilic bacteria in these three lakes – where salinity is low (1.18–6.82 psu) (Szmidt, 1972).

Donderski (1988) found that the proteolytic activity of bacteria isolated from the eutrophic Lake Jeziorak was 2–3 times higher than that in the mesotrophic Lake Jasne; it thus appeared dependent on the trophic status of the water basin. Little *et al.* (1979) showed that the degree of proteolytic activity may differ within the same basin. In Lake Champlain they found that the intensity of bacterial proteolysis was four times higher in its eutrophic part than in its oligotrophic part. This was confirmed by our present findings: the mean proteolytic activity was much higher in the hypertrophic Lake Jamno than in the eutrophic Lakes Łebsko and Gardno.

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